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IN VITRO DEVELOPMENT OF INSECT TISSUES. I. A MACROMOLECULAR FACTOR PREREQUISITE FOR SILKWORM SPERMATOGENESIS [1]

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Blood of metamorphosing silkworms contains a "macromolecular factor" (MF) which is indispensable for the maturation of their spermatozoa. This fact was documented nearly twenty years ago (Schmidt and Williams, 1953) in an investigation in which germinal cysts containing primary spermatocytes were removed from the testes of diapausing Cecropia or Cynthia pupae and cultured in hanging drops of hemolymph. In the absence of MF, the spermatocytes survived but failed to develop. By contrast, when MF was present in the culture medium, meiosis began within 24 hours and was followed by the rapid differentiation of spermatids and spermatozoa within the elongating germinal cysts. MF proved to be an undialyzable, non–species-specific factor which was stable at 75° C but rapidly inactivated at higher temperatures.

In assays of hemolymph removed from male or female Cecropia silkworms at successive stages in metamorphosis, Schmidt and Williams (1953) detected large and systematic changes in the titer of MF. For example, little or no activity was encountered in the blood of fifth instar larvae or of diapausing pupae. Substantial activity was detected during and immediately after pupation and, months later, during the termination of diapause and initiation of adult development.

Since virtually all aspects of insect metamorphosis including *in vivo* spermatogenesis were known to involve a hormone secreted by the prothoracic glands, Schmidt and Williams (1953) suggested that MF might constitute that hormone. This suggestion became untenable a year later when Butenandt and Karlson (1954) isolated ecdysone—a heat-stable, dialyzable steroid which, on injection into immature insects, provoked all the *in vivo* developmental phenomena previously realized by the implantation of living active prothoracic glands. Among these phenomena was the swift initiation of spermatogenesis when diapausing saturniid pupae were injected with ecdysone.

Yet, strange to say, ecdysone when added to the culture of germinal cysts proved completely ineffective in provoking *in vitro* spermatogenesis or in substituting for MF (C. M. Williams, as quoted by Karlson, 1956, page 248). Moreover, in unpublished experiments which Williams carried out in collaboration with Karlson, no evidence could be found for a complexing of ecdysone with any MF-like protein.

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For these several reasons, MF became and has remained something of an enigma.

During the past three years we have undertaken a detailed reexamination of *in vitro* spermatogenesis with special reference to the role of MF. In the present report we describe the production of MF, its changing concentration in the hemolymph of diapausing pupae, and its extraction and partial purification.

Materials and Methods

1. *Experimental animals*

Most experiments were performed on diapausing pupae of *Samia cynthia* derived from larvae reared on ailanthus trees under short-day conditions. The cocoons containing the pupae were stored at 25° C under which condition the diapause persists indefinitely. A few experiments were performed on three other species—namely, *Antheraea polyphemus*, *A. mylitta*, and *Hyalophora cecropia*. These species were reared or purchased from dealers.

2. *Preparation and evaluation of tissue cultures*

The technique of Schmidt and Williams (1953) was adapted with minor modifications. Groups of No. 1 coverslips wrapped in aluminum foil were heated overnight at 140° C. All other glassware was boiled in aqueous detergent ("Alconox," Alconox, Inc., New York) for 20 minutes, rinsed several times in tap water and distilled water, submerged for 10 minutes in 70% ethanol, and heat-dried at 140° C. Each item was then individually wrapped in foil and again heat-sterilized at 140° C overnight. Metallic instruments were soaked in 70% ethanol for 20 minutes and wiped dry with sterile tissue.

The pupae were surface-sterilized by submerging them for two minutes in 0.05% mercuric chloride in 50% ethanol, followed by several rinses in sterile distilled water. To collect samples of blood, a V-shaped incision was made at the tip of one or both forewings and the hemolymph was expressed into a chilled, sterile centrifuge tube containing a few crystals of phenylthiourea (PTU) to inhibit tyrosinase activity. The blood was centrifuged at 4° C for 15 minutes at 17,600 *g* and decanted into a sterile tube containing a few crystals of PTU. Hemolymph treated in this manner will be referred to as "plasma" in contradistinction to uncentrifuged "whole blood" containing hemocytes.

Pairs of testes were dissected from male pupae, placed in sterile disposable Petri dishes, separated from fragments of attached fat body, and rinsed in sterile insect culture medium (Grace, 1962). They were transferred to a second sterile dish, again rinsed in Grace's medium and teased open in a depression slide containing 200 μl of the desired culture medium. The testicular walls were discarded and the suspension of germinal cysts was subdivided onto six sterile coverslips. The latter were inverted and sealed with melted wax above the concavities of depression slides. Each culture contained 50–100 cysts in the volume of 30–45 μl medium.

The cultures were immediately examined under a compound microscope at 100 ×. As a rule they contained cysts arrested at the pachytene stage of the first meiotic division; a small percentage was at the earlier spermatogonial stage. In the rare instances where any cysts contained spermatids, the preparations were discarded.

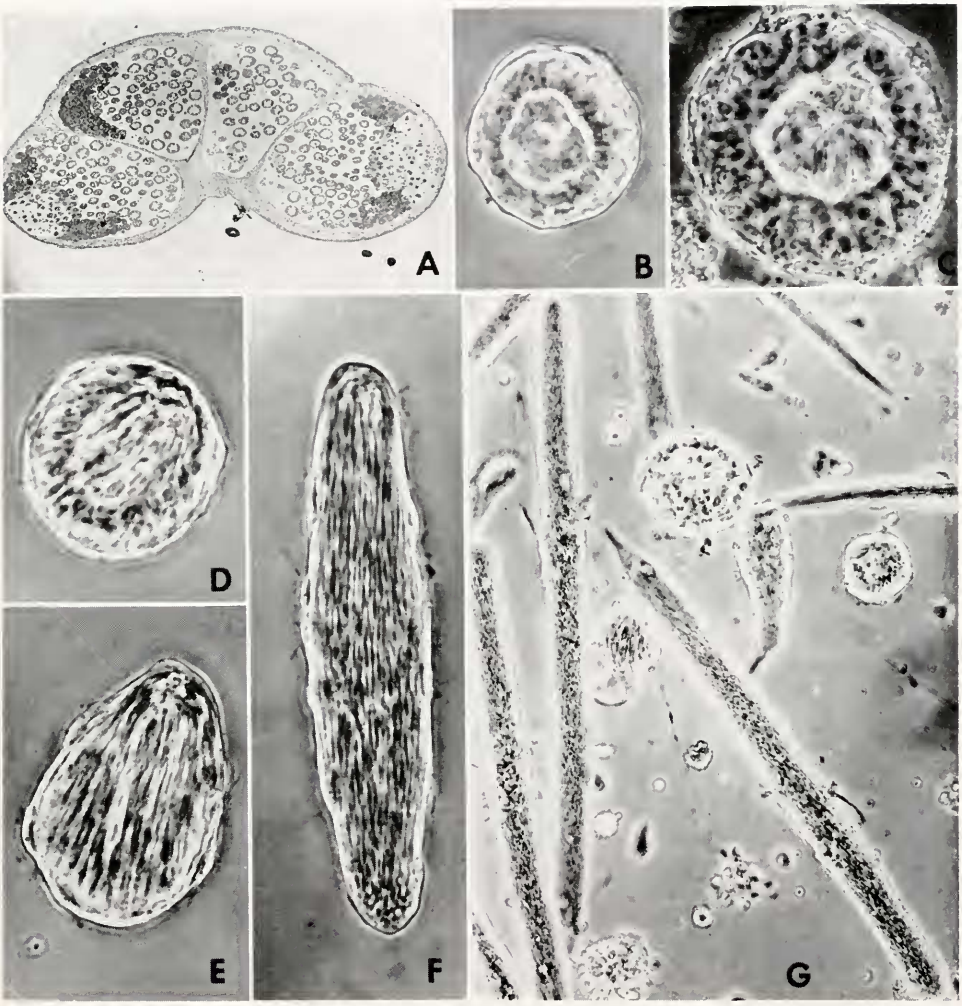

FIGURE 1. With the exception of Figure 1A (which is a section of a fixed and stained preparation), all other half-tones here and in Figure 4 are of phase contrast photographs of living cultures. (A.) Section of the testis of a diapausing Cynthia pupa; the germinal cysts occupy the four chambers formed by the inflections of the inner layer of the testicular walls (40 ×). (B.) A typical germinal cyst removed from a diapausing Cynthia testis. The primary spermatocytes surround the central lumen and are enveloped in a thin layer of follicle cells; Stage I according to the terminology of Schmidt and Williams (1953) (470 ×). (C and D.) Cysts after 24 hours of culture in MF-containing medium, meiosis has been completed and the axial filaments are oriented to the center of the lumen, Stage II (C = 660 ×; D = 470 ×). (E.) The spermatids and the cyst as a whole have begun to elongate, Stage III (470 ×). (F.) The cyst is now more than twice as long as wide, Stage IV (490 ×). (G.) Typical appearance of cultures after 7 days in MF-containing media. The very elongate cysts contain bundles of fully developed sperm. The less elongate were formed from cysts containing spermatogonia when the culture was prepared (160 ×).

The cultures were placed in a dark incubator at 25° C. Every 24 hours for a period of 15 days, they were reexamined. The cysts in each culture were counted and their developmental condition scored according to the classification of Schmidt and Williams (1953) (Fig. 1). The scoring was repeated for each culture and the average number of cysts in each stage was calculated each day. Cysts in developmental stages III and IV (E and F in Fig. 1) were scored as "positive"; these were summed and used to calculate the percentage of developing cysts which was used as a measure of MF titer. This calculation was ordinarily made on the tenth day.

3. *Culture media*

Most cultures were prepared in PTU-treated plasma or whole blood obtained from male or female pupae at specific stages in development. Certain cultures were prepared in "Grace's insect TC medium without insect hemolymph" (Grand Island Biological Co., Cat. No. 159). One series of experiments made use of three mammalian blood sera purchased from G.I.B. Co.—namely, Cat. No. 617, Calf serum; Cat. No. 614, Fetal calf serum; and Cat. No. 601, Newborn calf serum.

Undiluted insect blood or plasma is resistant to infection and it was not necessary to take any additional sterile precautions. However, when other media were utilized, the cultures were prepared in a UV-sterilized, plastic glove-box.

Osmolarities were estimated by the freezing-point method using a Model G-62 Fiske osmometer.

Results

1. *MF activity in the plasma of diapausing Cynthia pupae*

Newly spun cocoons of the Cynthia silkworms were stored at 25° C under 12 hours of daily illumination. At successive intervals 5 to 10 pupae were removed from their cocoons and bled. Each sample of blood was centrifuged and the undiluted plasma used to prepare a minimum of 30 hanging-drop cultures of germinal cysts derived from the testes of diapausing Cynthia pupae. The developmental responses were scored as described under METHODS and averaged for each group of cultures.

The results summarized in Figure 2 reveal large and systematic changes in MF activity in the plasma of both male and female pupae. The curve shows three self-evident phases: (1) a rapid decline during the first 8 weeks after pupation; (2) zero activity from the 8th to the 10th week; and (3) a reappearance of activity on the 11th week and a progressively increasing titer thereafter. The experiment was twice repeated with the same results.

These large changes in MF are of special interest because they take place in pupae which show no trace of the termination of diapause. In this sense the present results are not in full accord with those reported by Schmidt and Williams (1953).

2. *Inability of ecdysone to substitute for MF*

Twenty-one cultures of germinal cysts were prepared in the plasma obtained from diapausing Cynthia pupae after 2 to 3 months at 25° C. To twelve of these cultures α- or β-ecdysone was added in a final concentration of 1 μg per 100 μl

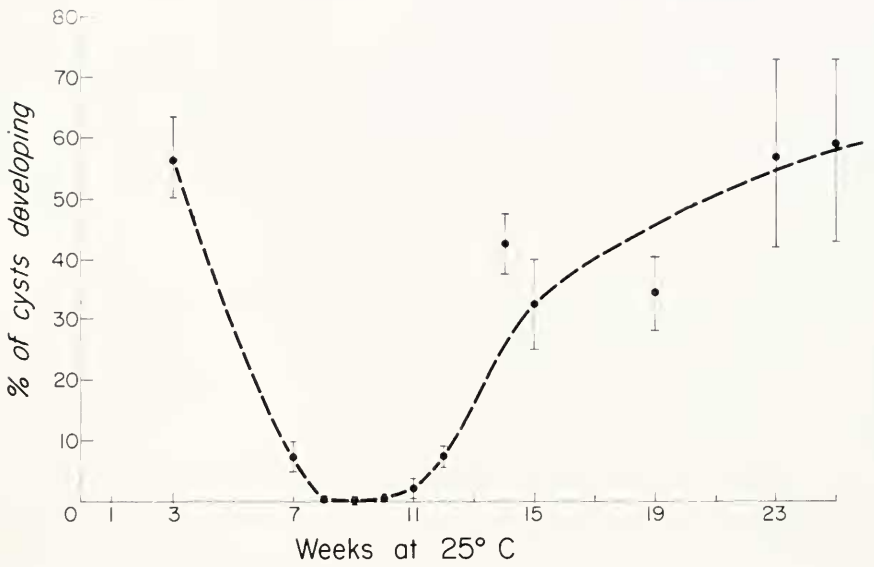

FIGURE 2. Changes in MF activity in the blood plasma of uninjured diapausing Cynthia pupae stored at 25° C. The vertical lines indicate the range of the measurements.

medium. The results as summarized in Table I reveal that the low but finite MF activity of the blood was not enhanced by the addition of ecdysone. The experiment was repeated on the plasma of pupae previously stored at 25° C for 4 to 5 months. Here again, the presence of ecdysone was inconsequential.

In an additional experiment 40 cultures were prepared in Grace's medium. In this case some of the cysts survived for 3 or 4 days but showed no trace of development in either the presence or absence of ecdysone. The rest of the cysts showed progressive dissociation into free spermatocytes and follicle cells all of which soon died.

These findings therefore confirm the previously cited conclusion that ecdysone is inactive in the *in vitro* spermatocyte assay and cannot substitute for MF.

TABLE I

Culture of Cynthia cysts in blood plasma of diapausing Cynthia pupae or in Grace's medium; ineffectiveness of ecdysone in provoking spermatogenesis

Culture media	Ecdysone not added		Ecdysone* added	
	No. cultures	% cysts developing**	No. cultures	% cysts developing**
Blood plasma:				
From pupae 2 to 3 months at 25° C	9	8 ± 4	12	3 ± 1
From pupat 4 to 5 months at 25° C	9	25 ± 6	12	26 ± 4
Grace's medium	20	0	20	0

* Either α- or β-ecdysone added in final concentration of 1 μg per 100 μl medium.

** Here and in subsequent Tables the mean and its standard error are recorded.

3. *In vivo activation of the plasma*

In an unpublished study which he carried out as a Harvard undergraduate, Dr. Robert D. Yee, of the University of Rochester School of Medicine, found that high titers of MF were routinely encountered in the blood of diapausing pupae after integumentary injury. We have confirmed Yee's important finding in the extensive series of experiments summarized in Table II. In these experiments diapausing pupae of three different genera were stored at 25° C for specific periods and then treated in the following manner:

An initial sample of blood (*ca.* 200 μl) was collected from each pupa. The sample was centrifuged and assayed for MF in order to determine the initial activity prior to injury. Each pupa was anesthetized with carbon dioxide for 25 minutes; it then received a large integumentary injury consisting of the excision of the tip of the abdomen which was sealed with a plastic window (Williams, 1959). After storage at 25° C for 24 hours the blood of each pupa was collected, centrifuged, and the resulting plasma assayed in the usual way.

The results summarized in Table II reveal a spectacular increase in MF activity in response to integumentary injury. Particularly impressive is the result obtained on the Cynthia pupae whose plasma showed zero MF prior to injury and a titer of 43% after injury.

4. *Dynamics of injury response; effects of hemocytes*

To clarify the time-course of the MF response to injury, the following experiment was carried out on a homogeneous group of 18 diapausing Cecropia pupae. Two hundred μl of hemolymph were first collected from each individual. Each

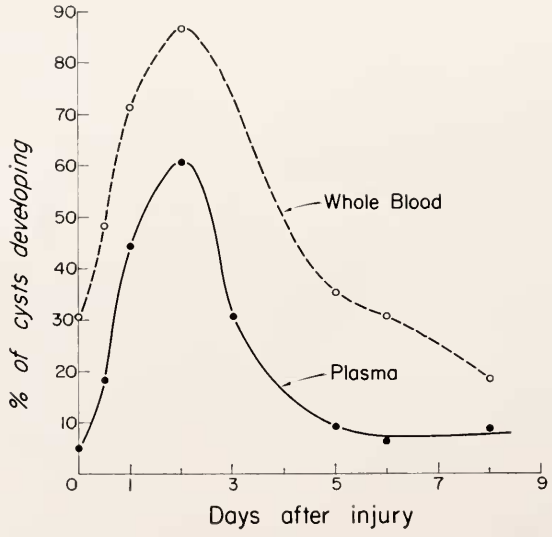

FIGURE 3. Changes in MF activity in the hemolymph of diapausing Cecropia pupae which were injured and then stored at 25° C. The upper curve is for assays on uncentrifuged blood; the lower curve is for blood plasma lacking any blood cells.

then received a large integumentary injury consisting of the excision of the tip of the abdomen, the wound being sealed by a plastic window. Two individuals were immediately sacrificed and bled. The remainder were stored at 25° C; at predetermined intervals two additional pupae were sacrificed and bled. All samples of blood including the initial samples were subdivided into two aliquots, one of which was centrifuged to remove the blood cells. The MF activities of all samples were assayed in the usual way on germinal cysts of Cynthia. Altogether, a total of 93 hanging-drop cultures were prepared from plasma and 89 from whole blood.

As indicated in the lower curve of Figure 3, the activity of the plasma was uniformly low in all pupae prior to injury (average 5%; range 0 to 8%). Within 12 hours after injury the plasma already showed a substantial increase in MF. The activity became maximal on the second day; it then underwent exponential decay during the following four days to the levels approximating those prior to injury. The upper curve in Figure 3 summarizes equivalent data for cultures prepared in uncentrifuged blood. Even the initial samples showed substantial MF activity ($30 \pm 6\%$). Here again, the activity was maximal in pupae sacrificed two days after injury.

In many additional experiments performed on Cecropia and Cynthia we have routinely found substantially greater MF activity in cultures containing whole blood rather than plasma. Evidently, the living hemocytes are able to contribute MF to the cultures over and above that already present in "injured" plasma. A direct test of this hypothesis was carried out in the experiment that follows.

5. *MF from cultured hemocytes*

Sixteen diapausing Cynthia pupae were utilized 7 months after pupation. Each individual received a large integumentary injury (excision of the tip of the abdomen and resealing with a plastic window). Seventy-two hours later the pupae were maximally bled into chilled centrifuge tubes containing a few crystals of an equal part mixture PTU and streptomycin sulfate. The hemocytes were collected by centrifugation (5 minutes at 3000 *g*). The plasma was discarded and the cells were washed three times in successive volumes of Grace's medium (total of 10 ml). Each of the 16 preparations of hemocytes was then resuspended in 2.5 ml of Grace's medium and placed in a 30 ml plastic culture flask (Falcon) at 25° C.

Within the first hour the hemocytes adhered to the plastic; they began to proliferate and within 72 hours had coated the plastic surface as a monolayer. This impressive multiplication (Fig. 4A–C) is of special interest since these blood cells constitute the only cell type which we have been able successfully to culture in Grace's medium unfortified with plasma or other macromolecules. This fact in itself suggests that the hemocytes are able to condition the medium by releasing macromolecules into it.

In the experiment in question the germinal cysts from two testes of Cynthia pupae were introduced into each culture after 24 hours. In 15 of the 16 cultures many of the cysts underwent meiosis and the initiation of spermatogenesis within one to two days. After three days, 10 to 70% of the cysts showed the positive assay for MF.

This clear-cut response was therefore impressively different from the absence of development and, in fact, the death within 3 to 4 days of germinal cysts cultured in

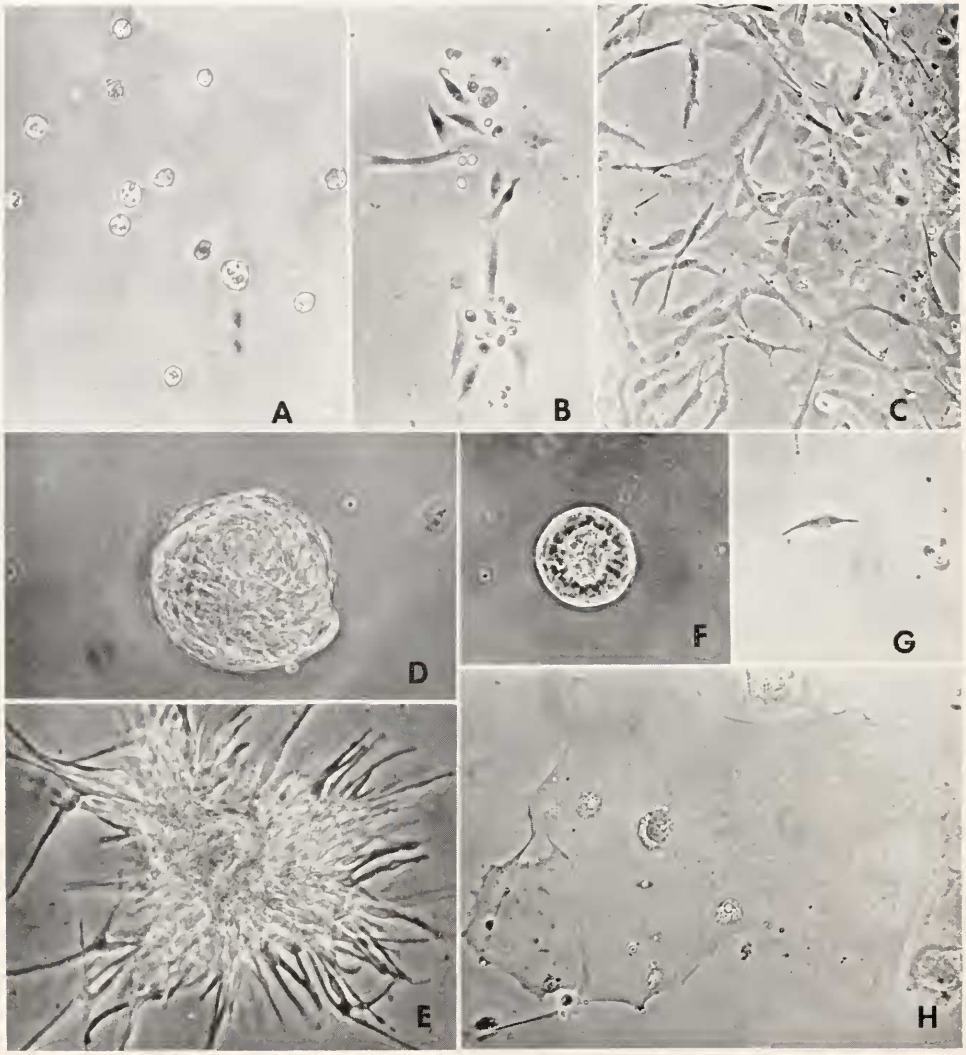

FIGURE 4, A.–H.

FIGURE 4 (A.–C.). Blood cells of previously injured Cynthia pupae cultured in Grace's medium, A. is after 15 minutes (490 ×), B. after 1 hour (290 ×) and C. after 7 days (290 ×). (D.) A developing cyst cultured for 2 days in Grace's medium which had been preconditioned for 24 hours by blood cells, the latter remaining in the culture. The developing sperm forms whorls within the non-elongating cyst (490 ×). (E.) A developing cyst as in Figure 4 D. except that in this case the cyst has spontaneously ruptured to reveal the differentiating spermatozoa (490 ×). (F.–H.) These three figures are reproduced at the same magnification (290 ×). F. is a normal germinal cyst from a diapauisng Cynthia testis. When subjected to osmotic shock, it dissociated into spermatocytes and follicle cells which were cultured for 10 days in Grace's solution containing a Cynthia plasma fraction precipitated by 3 M ammonium sulfate. The isolate spermatocytes (Fig. 4G.) survive but do not develop. By contrast, the isolated follicle cells proliferate and form giant cells illustrated in Figure 4H. For further details see text.

Grace's medium lacking plasma or hemocytes. Nevertheless, the development was abnormal in that the maturing cysts failed to undergo the elongation routinely encountered in cultures prepared in blood or plasma. They remained as spherical objects with the tails of the spermatids and spermatozoa forming whorls within the enveloping follicle cells (Fig. 4D and E). We suspect that this faulty development can be attributed to a failure of the medium to sustain the normal growth response of the follicle cells.

6. *MF in extracts of pupal fat body*

To determine whether other tissues contained MF, a series of Cynthia pupae 1 to 3 months after pupation was sacrificed and dissected in cold Grace's medium containing crystals of PTU. Masses of fat body were removed and thoroughly washed in the cold medium to remove nearly all hemolymph and hemocytes. The tissue was gently blotted, weighed, and approximately 1.5 g placed in 0.5 ml of cold Grace's medium in an all-glass homogenizer. After homogenization and centrifugation the clear supernatant was collected from between the precipitate and a superficial lipid layer. The precipitate was reextracted several additional times in small volumes of medium until a total of 2.5 ml of clear supernatant had been collected. The latter was sterilized by pressure-filtration through a Millipore filter (0.45 μ pore size) and placed in a 30 ml plastic culture chamber to which were added the germinal cysts obtained from two diapausing Cynthia testes. In a total of eight cultures of this type the cysts survived for at least six days but showed no development.

The experiment was repeated on a second group of Cynthia 1 to 3 months after pupation. In this case each pupa received a large integumentary injury to provoke the appearance of MF in the blood. During the first three days after injury the fat body of these individuals continued to show no detectable MF. However, by the sixth day after injury, considerable MF activity could be extracted from the fat body.

An additional experiment was performed on Cynthia pupae stored for 6 to 9 months after pupation. It will be recalled that the blood of these individuals contains high titers of MF (Fig. 2). So did their fat body when the latter was extracted and assayed in 5 of 6 cultures (20 to 70% development). By contrast, the intersegmental muscles and wing epidermis showed no extractable MF.

TABLE II

In vivo activation of the blood plasma of three species of diapausing pupae

Species of pupae donating plasma	Duration of diapause (weeks at 25° C)	No. pupae	Initial titer of MF	Titer 24 hours* after injury
S. cynthia	8	5	0 (10)**	43 ± 5 (16)**
H. cecropia	8	5	7 ± 7 (13)	55 ± 16 (7)
S. cynthia	15	6	27 ± 9 (27)	76 ± 12 (25)
A. mylitta	16	5	24 ± 6 (9)	78 ± 9 (9)

* All assays were on cysts of diapausing *S. cynthia* pupae.

** Number of cultures are recorded in parentheses.

From these preliminary studies it appears that, once MF is released into the blood, a certain proportion can be taken up by the fat body and sequestered.

7. MF-like activity in mammalian sera

In the case of insects as well as vertebrates the successful *in vitro* culture of cells and tissues in "synthetic" media has routinely required the addition of one or more macromolecular fractions (Wyatt, 1956; Eagle, 1955). For example, Grace's medium has generally been supplemented by 3 to 5% heat-treated (60° C for 5 minutes) hemolymph derived from diapausing silkworm pupae (Grace, 1962; Grace and Bryostowski, 1966)—a blood fraction which most likely contains substantial MF. Subsequently, it was found that hemolymph could often be replaced by other proteinaceous materials such as heat-treated calf serum (for detailed review see Brooks and Kurtti, 1971).

We were therefore encouraged to examine three commercially available mammalian sera for the presence of MF activity. Calf serum, fetal calf serum, and newborn calf serum (see section on METHODS) were heated at 56° C for 30 minutes to eliminate toxic components. The supernatants were then added in various proportions to Grace's medium and assayed for MF activity in plastic flasks containing germinal cysts derived from diapausing Cynthia pupae.

The results summarized in Table III reveal the surprising fact that all three mammalian sera stimulated the initiation of spermatogenesis when present in critical concentrations distinctive of each material. Meiosis took place accompanied by the beginning of elongation. However, development did not proceed beyond stage III and all cysts died and disintegrated after 7 or 8 days of culture. These findings suggest that the vertebrate sera contain one or more MF-like materials which cannot fully substitute for the authentic MF of silkworm blood.

8. Precipitation of MF at low ionic strengths

We have confirmed the unpublished findings of Dr. Melvin M. Ketchel (Tufts University Medical School) that MF can be precipitated from active plasma by the addition of critical amounts of distilled water. In the experiment in question 0.5

TABLE III

Flask cultures of cysts in Grace's medium supplemented with mammalian sera or Cynthia plasma

Sera	Number of cultures	% of cysts developing* as a function of % serum or plasma				
		20	40	60	80	100
Calf serum	20	6 ± 1	34 ± 9	21 ± 4	0	0
Fetal calf serum	15	10 ± 3	40 ± 8	20 ± 4	0	0
Newborn calf serum	50	16 ± 2	15 ± 5	61 ± 8	0	0
Active Cynthia plasma	30	0	30 ± 3	65 ± 7	80 ± 8	80 ± 7

* Each datum records per cent of cysts developing to Stages II and III; in the mammalian sera development never proceeded beyond Stage III.

TABLE IV

Fractionation of active blood plasma

Ratio hemolymph/H_2O	Number of cultures	% of cysts developing	
		Supernatant*	Precipitate**
1:0	15	61 ± 5	—
1:10	20	40 ± 6	0
1:20	20	32 ± 8	0
1:40	20	12 ± 3	20 ± 5
1:50	20	0	55 ± 6

* Lyophilized and redissolved in Grace's medium.
** Redissolved in Grace's medium.

ml of active Cynthia plasma was placed in each of four tubes. Double distilled water was added in volumes of 10, 20, 40, and 50, respectively. The tubes were incubated at 25° C for 30 minutes and then centrifuged at 17,600 *g* for 25 minutes. The supernatants were decanted, lyophilized, and redissolved in 0.5 ml of Grace's medium. The precipitates were also dissolved in 0.5 ml of Grace's medium. The osmotic pressure of each solution was determined and small amounts of additional Grace's medium (osmolarity 335 milliosmols) was added to lower the pressure to approximately 395 milliosmols. Each solution was sterilized by pressure-filtration through a Millipore filter and used to prepare 15 to 20 hanging-drop cultures containing germinal cysts of Cynthia pupae.

As summarized in Table IV, MF remained soluble after the addition of up to 20 parts water, but was partially precipitated in 40 parts water and fully precipitated in 50 parts water. The active precipitate permitted full development of the cysts including their elongation. When it was examined by disc electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (pH 7.2, 7.5% polyacrylamide, 0.1% SDS), one faint band and four dense bands were evident.

9. *Ammonium sulfate fractionation*

As a second approach to the purification of MF, 5 ml of active Cynthia plasma were dialyzed at 2° C against 1 liter of 2 M ammonium sulfate whose pH was adjusted to 6.8 at 2° C by the addition of sodium hydroxide. After 12 hours the non-dialyzable fraction was collected and centrifuged (20 minutes at 17,600 *g*), the precipitate being collected and temporarily frozen at −25° C. Further dialysis of the supernatant was carried out step-wise against 2.5, 3.0, 3.5, and 4.0 M ammonium sulfate (pH 6.8 at 2° C).

To eliminate residual ammonium sulfate each of the five precipitates was dissolved in 1 ml of 0.01 M phosphate buffer, pH 6.8, and dialyzed at 2° C for 24 hours against three changes of the same buffer. Each non-dialyzable fraction was lyophilized and made up to 2.5 ml with Grace's medium. The pH was adjusted to 6.8 at room temperature and the milliosmolarity to 395. Each solution was pressure-filtered through a sterile Millipore filter and then placed in a plastic culture flask to which the germinal cysts from two Cynthia testes were added.

MF activity was recovered only in the fraction precipitated in 3.0 M ammonium sulfate. In the culture containing this fraction, 30 to 40% of the cysts formed spermatids and spermatozoa. But here again (as in the case of cysts cultured in Grace's medium containing hemocytes) the cysts remained spherical and failed to elongate, the sperm flagella forming whorls within the cavity formed by the surrounding follicle cells.

In several experiments the cysts were first subjected to osmotic shock by exposing them to Grace's medium for about 0.5 hour. (As mentioned in Section 8, this medium is hypotonic in the absence of added serum or plasma fractions.) When the dissociated spermatocytes and follicle cells were cultured in Grace's medium supplemented by the MF-containing ammonium sulfate cut, dissociated spermatocytes survived for up to twenty days but failed to develop. Meanwhile, many of the dissociated follicle cells adhered to the plastic and remained viable for about a week; during this period they showed little growth and no multiplication. It is of particular interest that isolated spermatocytes did not respond to MF.

In cultures containing the four other plasma fractions lacking MF activity, the cysts remained intact and apparently healthy for several days; they then progressively dissociated into spermatocytes and follicle cells. Here again, the free germinal cells remained apparently healthy for up to twenty days but showed no development.

The behavior of the free follicle cells differed among the several cultures. In the presence of the plasma fractions precipitated by 2.0 or 2.5 M ammonium sulfate, a few of the cells adhered to the plastic; they showed slight growth but no multiplication, and usually died after 7 to 10 days. By contrast, in the presence of the plasma fractions precipitated by 3.5 or 4.0 M ammonium sulfate, the vast majority of the free follicle cells spread out on the plastic, increased in number by mitotic divisions, and then underwent enormous growth and polyploidization to form giant, flattened cells which remained healthy for as long as two months (Fig. 4F–H). These particular fractions evidently contained one or more components with the ability to promote the growth and development of isolated follicle cells but not of the germinal cysts as a whole.

Discussion

The normal milieu of the germinal cysts is the fluid which fills the testicular cavities (Fig. 1A). Interposed between this fluid and the surrounding hemolymph are the numerous cellular and membranous components which comprise the walls of the testes. All these barriers are automatically eliminated when the testes are torn open and the "naked" cysts are subjected to *in vitro* culture. Under this circumstance, the metamorphosis of the germinal cysts into bundles of spermatozoa was found to depend, not on ecdysone, but on an undialyzable, heat-sensitive, non–species-specific macromolecular factor which we have called MF.

As indicated in Figure 2, the titer of MF in the plasma can be described by a U-shaped curve when diapausing male or female Cynthia pupae are stored at 25° C for up to six months. No MF was detectable in assays performed on plasma collected from uninjured pupae during a brief period 8 to 10 weeks after pupation. These systematic alterations in titer are presumably attributable to differential changes in MF synthesis, compartmentation, and inactivation.

In Sections 4 and 5 of the RESULTS we considered the circumstantial evidence that one or more types of hemocytes may be the source of MF. Assays performed on whole blood routinely showed higher MF titers than the corresponding plasma. Moreover, when the hemocytes were collected from injured pupae and cultured in Grace's medium, they not only survived and multiplied, but also contributed to the medium an MF-like activity which stimulated the maturation of spermatocytes.

In cultures of this sort the germinal cysts failed to elongate despite the differentiation of spermatids and spermatozoa. This same abnormal development was encountered in cultures prepared in Grace's medium supplemented with an MF-containing plasma fraction precipitated by 3.0 M ammonium sulfate (see Section 9). When tested in this same manner, certain other ammonium sulfate fractions lacked MF activity but provoked a spectacular growth response of follicle cells isolated from the germinal cysts. Evidently, in addition to MF, the plasma contains at least one additional type of macromolecule prerequisite for the normal development of germinal cysts.

The successful culture of insect cells and tissues, as reported in the literature, has routinely required the presence of insect hemolymph or certain other macromolecular fractions derived from mammalian sera. It is therefore of considerable interest that MF-like activities were demonstrated in assays of heat-treated calf serum, fetal calf serum, and newborn calf serum. We are presently investigating the relation of these active materials to the "serum factors" prerequisite for the culture of mammalian cells (Eagle, 1955; Puck, 1961; Temin, 1967; Todaro, Matsuya, Bloom, Robbins and Green, 1967; Holley and Kiernan, 1968; Paul, Lipton and Klinger, 1971).

Whether MF constitutes a single molecular species can be determined only after its further purification and characterization. Further purification is also necessary to decide whether MF functions at catalytic or substrate concentrations.

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Summary

1. "Naked" germinal cysts removed from the testes of diapausing Cynthia or Cecropia pupae and cultured *in vitro* undergo meiosis and spermatogenesis only when the culture medium contains a "macromolecular factor" (MF) which is present in insect blood. The factor in question is undialyzable, heat-sensitive, and interchangeable among the three genera of saturniid silkworms which were studied. Its partial purification was achieved by ammonium sulfate fractionation and by precipitation at low ionic strengths.

2. In appropriate experiments, ecdysone was found to have no obvious effect on cultures of naked cysts and was neither able to replace nor enhance the activity of MF in the *in vitro* assay.

3. MF is present in the blood plasma of both male and female pupae; its titer undergoes large and systematic changes when diapausing Cynthia pupae are stored at 25° C for up to six months.

4. In plasma collected from uninjured pupae, MF was routinely present except during a brief period 8 to 10 weeks after pupation. However, even in that case, substantial MF activity appeared in the blood during the first two days after an integumentary injury.

5. Circumstantial evidence is presented that MF is synthesized and secreted by one or more types of hemocytes when the latter are activated as, for example, by integumentary injury. Presumably because of their ability to secrete MF and thereby to condition the medium, the hemocytes were the only class of cell which could be cultured in Grace's medium without the addition of any macromolecules.

6. MF-like activities were demonstrated in assays of heat-treated calf serum, fetal calf serum, and newborn calf serum. It is not yet known whether these activities are related to the "serum factors" prerequisite for the successful culture of mammalian cells.

7. For these several reasons it is conjectured that MF or MF-like materials have heretofore been present in virtually all of the media which have sustained the successful culture of insect cells and tissues.

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